Characterization of pneumococcal isolates from patients in Ethiopia

Thesis submitted as part of the Master of Philosophy Degree in International Community Health

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Abstract

Background: *Streptococcus pneumoniae*, the pneumococcus, is major human pathogen that causes more than 820,000 deaths worldwide annually, especially in developing countries. The most diseases presentations are acute respiratory tract infection (ARTI), otitis media, septicemia and meningitis. Pneumococcal meningitis has a higher fatality rate than meningitis caused by other bacteria. On the basis of the capsular polysaccharide type of the cell wall, can be serotyped into more than 90 serotypes. It is a vaccine preventable disease, and several vaccines based on different combinations of capsular component have been developed. The serotype distribution of this organism differs by geographical areas and the prevalent serotypes in one region could not necessary be the basis for vaccine formulation for another. Thus, it is essential to know the epidemiology and prevalence of the different serotypes.

Methods: To characterize pneumococcal strains in Ethiopia among diagnosed for pneumococcal infection by the physician and referred to the clinical microbiology laboratory of selected health institutions in Addis Ababa and Gondar from July to December 2012. All samples were cultured on appropriate culture media and typical pneumococcal colonies were confirmed by biochemical tests. Antibiotics resistance test was done by E-test strips and interpreted according to European guidelines. Serotyping was done by Quellung reactions, and isolates were characterized by multilocus sequence typing (MLST) to determine phylogenetic classification. Culture negative CSF from pyogenic meningitis were tested by Real time PCR.

Results: A total of 460 samples were collected, sixty one pneumococcal isolates were identified and most of them from cerebrospinal fluid (CSF). 15 serotypes were identified, with serotype 1 (21%) being the leading followed by 19F , 20 (10%) and 14 (8%). In seven samples more than one serotype were identified. Predicted coverage of 10 valent vaccine is 44%. Twenty four isolates were intermediate resistant for penicillin and except, for one and three all of them were susceptible to ceftriaxone and chlorampheinicol respectively. Seven new sequence types were identified by MLST. From 140 culture negative CSF, 12 were positive for meningococcus 9 for pneumococcus and 2 were mixed.

Conclusion: This study has a contribution evaluating the current vaccine program in the country.

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List of Abbreviations

AIDS	Acquired immunodeficiency syndrome
Alt	Autolytic
AST	Antimicrobial susceptibility test
ATCC	American Type Culture Collection
C3b	Complement 3 b
CAP	Community acquired pneumonia
CL	Chloramphinecol
CSF	Cerebrospinal fluid
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide
EHNRI	Ethiopian Health and Nutrition Research Institute
EM	Erythromycin
E test	Epsilometer test
Hib	*Haemophilus influenzae* type b
HIV	Human immunodeficiency virus
IPD	Invasive pneumococcal disease
Lyt A	Pneumococcal autolysin
LP	Lumbar Puncture
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing

NIPH	Norwegian Institute of Public Health
Nm	*Neisseria meningitidis*
PBP	Penicillin binding protein
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccine
PG	Penicillin G
PPV	Pneumococcal polysaccharide vaccine
REK	Regional Committee for Medical and Health care Research Ethics
SERO	Scientific and Ethical Review Office
SSI	Staten Serum Institut
Spn	*Streptococcus pneumoniae*
ST	Sequence type
TC	Tetracycline
TI	Trans-isolate
TIGR	The Institute for Genomic Research
TS	Trimethoprim sulphamethoxazole
TX	Ceftriaxone
WHO	World Health Organization

Chapter one: Introduction

1.1. Pneumococcal disease

The Gram-positive bacterium *Streptococcus pneumoniae* (pneumococcus) is the main cause of multiple disease conditions such as community acquired pneumonia, meningitis, otitis media, endocarditis, osteomyelitis, pericarditis , pyogenic arthritis , soft tissue infection , early-onset neonatal septicemia, lower respiratory infection and bacteriemia , all over the world, but especially in developing countries (1). According to the World Health Organization , the organism is causing more than 800,000 deaths per year (2).

The pneumococcus is a normal nasopharyngeal inhabitant which can live without clinical manifestation on the host and causes disease occasionally (3). Pneumococci spread by air droplet from person to person through coughing or sneezing and close contact with individuals who are carriers or infected. Pneumococcal transmission can be influenced by social and health factors, such as crowding , season, and viral respiratory co-infection. During the winter season the prevalence of pneumococcal disease increases in most places, especially in colder areas. Young children who are under the age of five and attending day care center, as well as the elderly are more susceptible to pneumococcal infections. Pneumococcal outbreaks may occur, for example in crowded jails and nursing homes. Immune - compromised individuals with HIV/AIDS or other chronic medical conditions such as heart or lung diseases and asplenia are at high risk of acquiring pneumococcal infections (4, 5).

1.2 Classification of the genus *Streptococci*

a) The genus *Streptococci*

The genus *Streptococcus* includes a group of bacteria that are medically important as well as some commensal bacteria. They are Gram -positive cocci in chain or pairs, non motile, non spore former, facultative anaerobic, catalase negative and some are encapsulated. Fastidious they can grown on culture media supplemented with 5% sheep or horse blood under CO_2 atmosphere (6).

This group of bacteria is subdivided on the basis of their hemolytic characteristics on blood agar, serologically based on the specific carbohydrate or group antigen, fermentation and tolerance tests, and sequencing of the 16S rRNA gene. The diagnosis of Streptococci In the clinical bacteriology laboratory, the diagnosis of Streptococci relies on the breakdown of red blood cells (6, 7) . According to their hemolytic characteristics they can be grouped as beta-hemolytic streptococci, which lyse red blood cells completely, alpha-hemolytic or partial hemolytic, and gamma-hemolytic streptococci which are non hemolytic. Serologically, beta-hemolytic sreptococci can be differentiated by the presence of group specific antigens that might be C-carbohydrate or teichoic acid. By this classification, or Lancefield grouping, they can be grouped A to V. Bacteria such as *S. pneumoniae* and viridians group of streptococci that lack C-carbohydrate or M protein cannot be classified by this method. The ability to ferment carbohydrates and tolerance to salt concentration or pH change are also used for classification. The current and most reliable method of classification is by gene sequence analysis the resulting phylogenetic tree. Around 99 groups of streptococci are recognized on the basis of 16S rDNA sequences and the main ones include : *S. pyogenes*, *S. agalactae*, *S. equismilis*, *S. bovis*, *S. sanguis* , *S. swis* , Viridians *Streptococci.* The viridians *Streptococci* contains Mitis, Mutans, Salivarius and Anginosus (8).

b**) Phenotypic differentiation of S. pneumoniae**

S. pneumoniae belongs to the Mitis group. It is alpha hemolytic and has no group antigen. The bacterium measures 0.4 μm in width and 1 to 1.5 μm in length. It is the leading cause of pneumonia and the morphology of the organism, respectively. The organism needs catalase containing media, produces pneumolysin (formerly called haemolysin) which breaks down hemoglobin into a green pigment. As a result pneumococcal colonies are surrounded by a green zone during growth on blood agar plates. Fermentation of monosaccharides, (glucose, mannose, fructose and galactose), disaccharides, (sucrose, lactose, trehalose, maltose and cellobiose) trisaccharide, (raffinose) or a fructose oligosaccharide (inulin) can distinguish them from other species (8, 9, 10).

To differentiate *S. pneumoniae* from other viridians streptococci which have similar hemolytic character and belong to the normal human nasopharyngeal flora, optochin (ethylhydrocupreine) susceptibility test or bile solubility tests are used. A simple method is observing an inhibition

zone of 14 mm or more around an optochin disk. The bile solubility test is performed by observing the clearance of turbidity of sodium deoxycholate after two hours incubation at 37^{o}C. All suspected pneumococcus with inhibition zone of 10-13 mm diameter by optochin test should be confirmed by bile solubility test. Other viridians are negative in the optochin test and do not clear sodium deoxycholate after two hours incubation at 37^{o}C (9).

1.3 Cell wall composition and serotyping

Like other Gram positive organisms, the pneumococcus has a cell wall containing a peptidoglycan layer and teichoic acid in larger amount (Figure 1). The polysaccharide substance (Slime layer) around the cell wall secreted by the bacterium, becomes thick and forms a capsule. The cell wall is covered by a polysaccharide capsule that is immunogenic and its polysaccharide composition differs among strains. The capsular antigenic component of the organism is used to divide into serotypes.

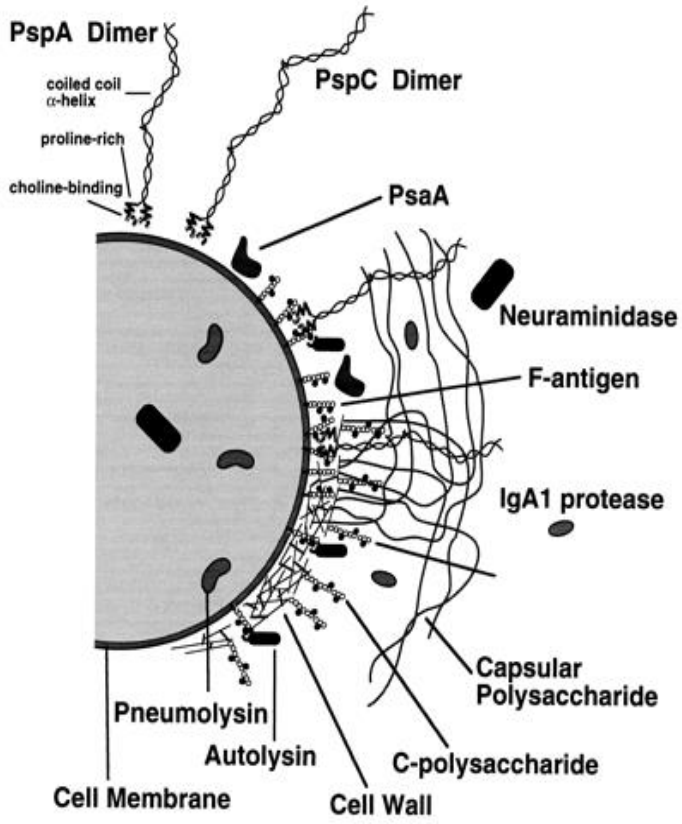


Figure 1 Pneumococcal cell wall (38)

1.4 Laboratory methods to verify and characterize *S. pneumoniae*

a) Standard methods for laboratory isolation methods of *S. pneumoniae*

Pneumococcus can be identified by different types of laboratory techniques. These include the simple and rapid technique of Gram stain to the complicated method of identification of the organism at the gene level. The simplest method is observing Gram positive cocci in pair under the microscope from a clinical sample. The gold standard is the culture method, isolating live organisms and doing further identification as described above and antimicrobial susceptibility test (9, 10).

b) Serotyping

The Quellung reaction is based on the swelling of the pneumococcal capsule. Upon binding with homologous antibody the capsule surrounding the bacterium swells and become visible under the microscope. Swelling and aggregation of cells indicates a positive reaction for the serotype specific antibody. On the basis of variation in capsular components, currently there are 94 serotypes identified (11) .

c) Antimicrobial susceptibility testing

A predefined antimicrobial gradient concentration on a plastic strip can be used to determine the minimum inhibitory concentration (MIC) of antibiotics. The antibiotic on the strip will diffuse in the agar plate on which bacteria are inoculated and inhibits the growth. After overnight incubation at 37^{o}C, MIC can be interpreted (12).

1.5 DNA based detection and characterization

a. Conventional PCR

This method is used for detecting bacterial pathogens from clinical samples if the culture test negative due to prior antibiotic treatment or cell lysis. To do this test, polymerase enzyme, primer or strand of DNA to start synthesis, dNTP which are building blocks of DNA and single or few copies of the targeted DNA are required. It has three steps; first the double strand DNA will be denatured and become single strand, followed by annealing of primes with complementary single strand of targeted DNA then extension of primers 5' to 3' direction by

polymerase enzyme and double stranded DNA will be produced. This cycle repeats many times and at every extension step DNA molecules would be doubled and finally thousands or millions of targeted DNA will be amplified. At the end of DNA amplification, the PCR product will be detected, visualizing under UV light running agarose gel electrophoresis (13).

b. Real Time PCR

Real time PCR is modified version of conventional PCR that detects based on fluorescent dies. This is also another molecular detection but more sensitive and fast detecting bacterial pathogens from clinical samples. It is a kinetic method which amplifies and detects targeted genes simultaneously. There are two types of RT-PCR: non- specific and specific ones. The non specific one uses fluorescence dye that intercalates in to any double stranded and emits light. The specific one uses probe which contains nucleotides labeled with fluorescent reporter which emits resonance energy to a quencher when it reacts with its complementary DNA target. The targeted gene is *lyt A* gene which is common for all clinical pneumococcal isolates (2, 14).

c. MLST

Multilocus sequence typing (MLST) is based on amplification and sequencing of seven housekeeping genes which are common for all clinical isolates of pneumococcus. This method is used to identify the genetic diversity of clinical isolates and their phylogenetic relationships (15).

1.6. Serotype distribution among *S. pneumoniae*

Out of the 94 serotypes identified, 20 to 25 are the commonest causative agents of pneumococcal infections. while the remaining ones usually live commensally (16). The distribution of the serotypes may differ in different geographical locations, season, immune status of the individual and age group of the host. The main clinical manifestations against different serotypes may also differ. (17)

***a)* Geographical distribution of serotypes**

The pneumococcal serotype distribution is different in industrialized countries and developing countries. Serotypes:14, 4, 1, 6A, 6B, 3, 8, 7F, 23F, 18C, 19F, and 9V are the most frequent in decreasing order all over the world and are together the cause for more than 80% of the global pneumococcal burden (16). In North America, the serotype distribution is (14, 6B/ A, 19F/ A, 18C / B/ F, 23F / B / A, 4, 7F, 3, 1, 15C / B/ A and 12. In Europe the serotype distribution is similar to that in North America with only a slight differences. In Africa the serotype distribution differs with in decreasing order : 6B/ A, 14, 1, 19F/ A, 23F, 5, 15,18F/ A, 4, 7F/ A, 9N/ L and 8. Serotype 14 causes most cases of invasive bacterial disease (IPD) followed by 6B and 6A (18).

A study conducted in the Gambia, West Africa, indicated that the commonly identified serotypes from IPD cases were 1, 5, 14, 6A, 19A, and 23F (18). Another study conducted in countries of the East African region by netSPEAR identified serotypes 1, 6B, 14, 6A, 23F, 19A as the most frequent (19).

b) Age

Children under the age of five and elderly above sixty are more vulnerable for pneumococcal infections. In developed countries pneumococcal infections are more prevalent in the elderly whereas in developing countries young children are more affected. The serotype distribution varies among different age groups. Serotypes 14, 6B, 1, 19F, 23F are more frequent among young children, with serotype 14 being the leading cause of pediatric pneumococcal infection. Serotype 1 is more isolated from children between the age of one and five than children below the age of 2 (17, 20).

1.7. Carriage of *S. pneumoniae*

S. pneumoniae has no environmental reservoir; and humans are its only reservoir. The organism colonizes the human nasopharynx within the first year of life. More than one serotype could colonize the same host at the same time and live together. the host may become a source for horizontal transmission or the carriage may end up to a year. The carriage rate is more prevalent in young children under the age of five who have poorly developed immune system. On the other

hand followed by the acquisition of the pneumococcus the host carrying the organism for a long time, may produce serotype specific antibodies and clear the organism within a couple of weeks (22). The carriage rate decreases in the adulthood period. The carriage rate can increases up to 40 to 60% in children and up to 10 to 15 % in adulthood (21).

After colonization in the upper respiratory tract the bacterium can cause local or systemic pneumococcal diseases. Clinical manifestations depend on the inoculums size and the virulence of serotypes (22).

1.8. Risk factors for pneumococcal infection

Both host and environmental factors can aggravate the infection of pneumococcus. The host factors associated with high risk for pneumococcal disease include: viral upper respiratory tract co-infection, abnormal epithelial cells at the upper respiratory tract, immune status (chronic disease, immune-compromised) and extreme age groups. Environmental factors include airborne pollutants like tobacco or indoor fire cooking and heating, crowded living condition as in jail or nursing home, and living in cold areas (23).

1.9. Pathogenesis, pathology and virulence factors

The portal of entry for the organism is through the upper respiratory tract of humans. It can cause respiratory infection in this niche, as well as migrate to a variety of niches and cause deprived types of disease including otitis media, community acquired pneumonia, septic arthritis, septicemia and meningitis. Around 25% of community acquired pneumonia is caused by pneumococcus; especially , it is the leading cause of pneumonia among HIV positive individuals. The organism has different mechanisms to colonize the host as well as to cause disease. Genetic and physical components of the bacterium play great role in pathogenesis (24).

The first step of pathogenesis is adherence to the host cell. The organism adhere to the mucosal epithelial cells of the nasopharnyx using N- acetyl galactosamine β 1-3 galactose (GlcNAcβ1-3Gal) disaccharide receptor on the host epithelial cell. The pneumococcal surface protein or choline binding protein A CbpA is exposed to the external surfaces and attaches with the

nasopharyngeal epithelial cells. Some non specific factors also enhance the adherence of the organism with the host receptor cells. These factors include hydrophobic interaction between cells of the host and the bacterial cell, surface charge and pH. (25)

1.10 Virulence factors

The pneumococcus possesses different virulence factors that are mandatory to escape the host immune response during infection. Pneumococcal infection and its complications result partly from the direct actions of pneumococcal virulence factors and the corresponding immune responses to various pneumococcal components resulting in adhesion, invasion, inflammation and shock . Pneumococcal virulence factors mainly include:

Table 1. Major pneumococcal virulence factors and their function

Virulence Factor	Function	Reference
IgA protease	Cleaves human IgA1, preventing antibodies from inhibiting adhesion.	26
Cell wall	Releases inflamatory response	10, 26
Surface protein (Pneumolysin)	Pore formation on host cell membrane, enhances pathogenicity	24
Hyaluranidase	Degrades connecting tissue	27
Capsular polysaccharide	Prevents the organism to be removed by innate immunity.	10, 26,29
Pilli	Binds with host cells	28

Capsular polysaccharide:.

The polysaccharide capsule is the main virulence factor, used to colonize the host and prevents the removal of pneumocccus by innate immunity. This also protects the organism from phagocytosis by leukocytes following opsonization inhibiting the C3b complement system. (10, 25, 26)

1.11. Treatment and antimicrobial susceptibility pattern

Pneumococcal infections can be cured by antibiotic treatment. Traditionally penicillin was the drug of choice. All patients diagnosed with pneumococcal disease were empirically treated by penicillin. (30) But after the first reports of penicillin resistant pneumococcus, in 1967, from Australia and then from Papua New Guinea and South Africa, resistance all over the globe become a problem and antibiotics other than penicillin had to be used. (31) Currently, multi drug resistant pneumococci are spreading all over the world and become a great public health concern. (32)

Improper taking of antibiotics without prescription and improper dose select for resistance. Prescribing antibiotics as a prophylaxis can also create antimicrobial resistance. A study conducted in Ethiopia, indicated that individuals who were treated by azithromycin for trachoma, have acquired resistant strains of pneumococcus, compared with placebo groups who did not. (33) The antimicrobial resistance could be by mutation or horizontal exchange of genes between non related bacteria via transformation . Often pneumococcal isolates from the respiratory tract have a high rate of antimicrobial resistance due to horizontal exchange from the nasopharyngeal flora. For resistance to penicillin the pathogen alters the penicillin binding site of the penicillin binding proteins which leads to low affinity of penicillin and other beta-lactam antibiotics. (2, 32)

The fatality rate of pneumococcal infections caused by resistant pneumococcus could reach up to 50% even under care. To avoid such problems treating pneumococcal infections according to the microbiological findings is mandatory for better therapeutic outcome or preventing the spread of resistant isolates through the community. Identifying the antimicrobial susceptibility pattern of

isolates would have a great role on reducing the fatality rate caused by the organism, as well as social and economic impact.(35)

Currently, the burden of antimicrobial resistance is very high all over the world including the developing countries. Antimicrobial resistance is specific for some strains of pneumococcus and serotypes 6A, 6B, 9V, 14, 19F, 23F are responsible for most of the resistance to penicillin and other related antibiotics. During the era of vaccine, another challenges is that strains not covered by the vaccine may replace vaccine strains become more resistant to antibiotics that are primary choices for the treatment of pneumococcal infection. (34, 36) .

1.12. Prevention

Pneumococcal infections are vaccine preventable, and polysaccharide vaccines have been developed. However, antibodies which are produced against one serotype are specific for that serotype and cannot induce immunity against other serotypes. The serotype distribution of this organism, differs by geographical areas and the prevalent serotypes in one region are not necessary by a vaccine designed for another region. To be effective the vaccine should contain serotypes that circulate in the specific community otherwise the outcome will not be that much significant. (10)

Different types of pneumococcal vaccines are produced and licensed. The first polysaccharide pneumococcal vaccine was produced around 1940s but licensed in 1977 and used in the United States first 1983. It contained purely polysaccharide capsule of 23 serotypes (1, 2 , 3 ,4 ,5 , 6B , 7F ,8 , 9N ,9V ,10A ,11A ,12F ,14 ,16B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F). This vaccine is used for susceptible groups, like elderly and immune- compromised individuals, but it is not immunogenic for children under the age of two.(37)

A seven - valent pneumococcal conjugated vaccine (PCV-7) was produced in the year 2000. This vaccine contains seven pneumococcal serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) which are the most prevalent in North America and Europe. This heptavalent vaccine covered more than 80% of the pneumococcal burden in the region. This vaccine was immunogenic enough to induce antibody in younger children due to the conjugated meningococcal protein. Introduction of

PCV-7 has limited value for developing countries, however, due to the lack of additional serotypes 1and 5 which are frequent in these nations. Including three more pneumococcal serotypes (1, 5, and 7F), the PCV-10 vaccine was produced in 2009 by Glaxo-Smithkline. Around 2010 , including another six more pneumococcal strains to PCV-7 (1,3, 5, 6A, 7F and 19A), the PCV-13 vaccine was produced and licensed (38, 39).

The PCVs have a great role in reducing the pneumococcal disease burden, as they are more immunogenic than the polysaccharide vaccine in all age groups. Especially, the PCV-13 may reduce antimicrobial resistance by preventing the more resistant strains, like 19A, and 6C (by cross protection). Currently, PCV-7 is replaced by PCV-13. The vaccine should be given to individuals in high risk groups. Before introduction of vaccine for intervention program, there should be evidence-based data which can determine the characteristics of pneumococcal isolates based on their capsular serotype which circulate in the community. The conjugated polysaccharide capsule should cover at least 60-80% of strains causing invasive disease (39, 40).

1.13 Pneumococcal disease in Ethiopia and rational for the study

a) Pneumococcal disease and vaccination program in Ethiopia

Ethiopia is located at the horn of Africa bounded by Eritrea to the north, Kenya to the south, Somalia and Djibouti to the east and Sudan and South Sudan to the west. It has a land area of 1,104,300sq km and with 85 milion people of more than 80 ethnic groups. The capital is Addis Ababa which is the largest city , with a population of more than 4 million. Most people live at the countryside, with only 17% urban population (41).

In Ethiopia communicable diseases are most common due to climatic variation, poor sanitary, and inaccessibility of health facilities, in spite of awareness. The child mortality rate 123/1000 (42). Currently more than 30,000 health extension workers are trained and deployed in rural areas. Their training is based on disease prevention policy. They are trained on health packages of maternal health, child health, reproductive health, and others to have a primary health service in the community.

Ethiopia is one of the countries severely affected by pneumococcal infections. However, although there are reports of clinically suspected cases of pneumococcal disease there are no laboratory- confirmed cases reported officially to the World Health Organization (WHO) (43) . Knowledge of serotype distribution and antimicrobial susceptibility patterns are important in relation to the treatment of pneumococcal disease and vaccination programs.

PCV-10 was introduced in 2011 for inclusion in national vaccination program although the serotypes that circulate in different communities were not known only two studies have previously reported on serotype distribution among Ethiopian pneumococcal isolates. (44)

A study was conducted at the Ethio-Swedish Children's Hospital in Addis Ababa from 1993 to 1995. The purpose of the study was to identify the prevalence of pneumococcal and *Haemophilus* serotypes and to study antimicrobial susceptibility pattern. In this study around 46 pneumococci were isolated and the coverage of existing pneumococcal vaccines was from 48 to 65%. This study however had limitations due to a small sample size, and the sample collection sites were not adequate. It was also performed two decades ago and more recent studies are mandatory in order to generalize for the entire country (35)

In 2009 there was a report pneumococcal burden in the East African region by The Network for Surveillance of Pneumococcal diseases in the East African Region (netSPEAR). The report includes 25 pneumococcal isolates from the Black Lion Hospital in Addis Ababa, but there was no serotype result due to lack of preserved live organism. The netSPEAR data includes only Kenya, Tanzania and Uganda. To fill the gap current and updated data are essential. (19)

b) Rationale for this study

Generating data on the Ethiopian pneumococcal serotype distribution and their antimicrobial susceptibility pattern can give appropriate information for policy makers. The data that can be obtained from this study will be components to strengthen the current pneumococcal immunization program or identify the gap. It would be part of the solution for current program or could be components for future studies.

Chapter two: Objective

2.1 General Objective

To determine the serotype of pneumococcal strains which circulate in Ethiopia and their antimicrobial susceptibility pattern.

2.2 Specific Objective

To isolate pneumococcal strains from clinical samples

To perform antimicrobial tests on pneumococcal isolates

To identify the serotype distribution among the pneumococcal isolates

To identify the genotype distribution among the pneumococcal isolates and relate to serotypes and antimicrobial susceptibility results

To provide information for policy makers relevant for drug use policy and vaccination programs

Chapter three: Materials and methods

3.1. Study design

The study design was cross sectional study.

3.2. Target population

The target population was all individuals who were clinically suspected for pneumococcal infection like meningitis, pneumonia, septicemia, especially children ≤59 months of age and elderly greater than the age of 60.

3.3. Study sites

The Black Lion (Tikur Anbessa) Hospital, Addis Ababa, is the largest teaching hospital for the University of Addis Ababa Medical School in Ethiopia, with about 800 beds. It is staffed by 130 specialists and 50 non-teaching doctors. The emergency department sees around 80,000 patients a year.

The University of Gondar Hospital, Gondar, Amhara region is a 400 bed university hospital, which acts as the referral centre for four district hospitals in the area. It has a range of specialties including pediatrics, surgery, gynecology, psychiatry, HIV care and an outpatient clinic. Within its 400 staff it employs 50 doctors, 150 nursing staff, three pharmacists, 90 care staff and 25 laboratory scientists. These staffs serve a population of four million across the region. As a university hospital, it plays an important role in teaching medical and nursing students.

Yekatit 12 Hospital is a referral hospital in the capital Addis Ababa. It gives service for clients who are referred from public or private health institutes in Addis Ababa. It has pediatrics, surgery, gynecology, psychiatry, HIV care and an outpatient clinic.

The St Paul Millennium Medical College Hospital is a specialized general hospital located in Addis Ababa, next to the Ethiopian Health and Nutrition Research Institute (EHNRI). It has pediatrics, surgery, gynecology, psychiatry, HIV care and an outpatient clinic. Usually, most microbiology tests from the hospital are referred to EHNRI.

The Addis Ketema Health Center and Wereda 7 Health Center are found in the capital city at the sub city of Addis Ketema. Selam Health Center is also located in the capital city at Gulele sub city. Each health center has around 250, 000 catchment population and provides an outpatient clinic, mother -child health service, and HIV care and follow- up for TB patients.

EHNRI is the national reference laboratory located at the capital city Addis Ababa. It conducts research on infectious and non infectious diseases, nutrition and food science, traditional medicine, regional laboratory capacity building and diagnostic laboratory service for patients who are referred from public and private health institutes.

Study sites were selected according to pediatric facilities (Senior pediatrician) as well as laboratory test available (microbiology culture test availability or vicinity to the microbiology laboratories).

Figure 2 Map that shows study sites in Ethiiopia (45)

3.4. Sample size and sampling strategy

By convenient sampling method all individuals with sign and symptoms of pneumococcal disease who visited these health facilities during the study period were included in the study. All pediatrics CSF suspected for bacterial meningitis from all study sites were included in the study.

Table 2 Sample type and their collection history

Sample type	History and method for sample collection	Collection site
CSF	Suspected meningitis cases of pediatrics unit	The PBM* study in EHNRI
Blood	Suspected septisemia	Black Lion
Throat swab	Suspected of pneumonia	Selam Health Center St Paul and Wereda 7
Sputum	Suspected of upper respiratory tract infection	St Paul Hospital, Addis Ketema, Wereda 7 and Selam Health Centers
Pleural fluid	Suspected of arthritis cases	Black Lion and St Paul Hospital
Ear swab	Pediatic otitis media infection	St Paul Hospital and EHNRI

* PBM= Pediatric bacterial meninigitis surveillance conducted by WHO in collaboration with EHNRI.

3.5. Sample collection period

The collection period was between July 2012 to December 2012.

3.6. Inclusion Criteria

All patients who have been diagnosed for pneumococcal infection by the physician and referred to the clinical microbiology laboratory were eligible for entry into the study.

3.7. Sample collection

CSF samples were collected by lumbar puncture performed by a well -trained physician. Drops of CSF were collected into sterile screw cap tubes with 1 ml of CSF fluid, the minimum required, and 3 to 4 ml desirable. Within 1 hour of collection the samples were taken to the microbiology laboratory. Samples collected outside of the working hour were also inoculated into Trans Isolate (TI) media, which is diphasic and can preserve fastidious organisms for a long time (2, 46).

Culture negative CSF samples from the university of Gondar were collected in nunc tube without preservative and stored at -70^{o}C until transport to Norwegian Institute of Public Health (NIPH).

Throat samples were collected using sterile cotton tipped swabs with Amies charcoal transport media, produced by Oxoid. For babies, the mother made the child to open the mouth while the physician took the throat sample. The samples were transported to the laboratory within a day of collection.

Blood cultures were collected by well trained laboratory technicians or physicians. The blood was drawn up to 10 ml for adults and 1-3 ml for children. Then, aseptically disinfecting the cap of the bottle with 70% alcohol, the blood was added to thioglycolate medium in ratio 1:5 and mixed gently by inverting the bottle.

Pleural fluids were aspirated by well - trained physicians using 18 guage needle with a 10 ml syringe.

Sputum samples were collected by the patients themselves. They were advised to collect morning deep coughing, purulent sputum without saliva in a sterile screw caped sputum cup.

Middle ear discharges were collected using commercially prepared sterile cotton tipped swab with Amies charcoal transport media. The physician took the discharge using sterile cotton tipped swab and dipped into the charcoal to transport to the laboratory (46, 47).

3.8 Identification of the agent

3.8.1. Culture

As soon as the samples arrived to the laboratory, CSF and pleural fluids were centrifuged for 20 min at 2000 rpm and the supernatants were removed with a Pasteur pipette and saved for serology test. One or two drops of the sediment was used to prepare a Gram smear and 1 to 2 drops to streak onto the primary culture media (5% sheep blood agar and chocolate agar supplemented with Isovital X). For CSF samples with less than 1 ml culturing and Gram stain were made directly without centrifugation. (2)

Blood culture bottles were incubated at 37^{o}C, and after overnight incubation, Gram smear and subculture onto 5% sheep blood agar were done. Blood culture bottles were incubated further for seven days inspecting turbidity visually every day. Finally sub-cultivation was performed at the seventh day.

The streaked plates were incubated under 5 to 7% CO_2 concentration (candle jars). After overnight incubation *S. pneumoniae* isolates were identified by colonial morphology and alpha hemolysis. Typical colonies of pneumococcus were confirmed by optochin susceptibility test with inhibition zone of 14 mm or above and bile solubility test. (2, 9, 10, 17) Colonies that were confirmed by optochin susceptibility and bile solubility tests were put in a vial of Greave's solution prepared at NIPH. The vials were stored at -80^{o}C at EHNRI until transport to NIPH. Culture negative CSF n= 196 and preserved pneumococci n = 61 were sent to NIPH by World Courier using dry ice. At NIPH the preserved vials were sub-cultured on 5% sheep blood agar and incubated overnight at 5% CO_2.

After overnight incubation fresh colonies were picked up for antimicrobial susceptibility test, serotyping by Quellung test, and DNA preparation for MLST (15, 48, 49)

3.8.2. Antimicrobial susceptibility tests

MIC of antibiotics were determined by the E-test method which uses graduated concentration of antibiotic on a strip. The culture medium for antimicrobial susceptibility test was Muller Hinton Fastidious, supplemented with 5% sheep blood and 20 µg β-NAD. A loopful of pure pneumococcal culture was inoculated in Muller Hinton broth and cell density of the suspension was measured with a spectrophotometer at 0.5 McFarland Standard. Then the medium was

inoculated using a sterile cotton swab by the machine for even distribution on the plate. Antimicrobial strips were put on the media automatically by the machine and incubated into 5% CO_2 at 37^{o}C overnight. After overnight incubation the MICs were observed and interpreted with 80% colony inhibition for bacteriostatic antibiotics and clear zone for bacteriocidal antibiotics. The antibiotics tested were penicillin G (PG), ceftriaxone (TX), erythromycine (EM), tetracycline (TC), chloramphenicole (CL) and trimethoprim -sulphametoxazole (TS). Penicillin, ceftriaxone and chloramphinicol are bactericidal and the remaining were bacteriostatic. The above minimum inhibitory concentration (MIC) results were interpreted according to the European committee on antimicrobial Susceptibility testing guideline (53). Pneumococcal isolates with clear inhibition zone of ≤ 0.06 µg/ml of penicillin indicates susceptibility to penicillin and pneumococcal isolates of MIC ≥ 0.12 µg/ml ≤ 2 µg/ml as intermediate resistant and > 2 µg/ml are resistant for penicillin. Ceftriaxone pneumococcal isolates with MIC of ≤ 0.5 µg/ml and are susceptible and > 2 µg/ml are resistant. Erythromycin pneumococcal isolates with MIC ≤ 0.25 µg/ml are suceptible and > 0.5 µg/ml are resistant. Tetracycline pneumococcal isolates with MIC ≤ 1 µg/ml are suscptible and > 2 µg/ml are resistant. Chloramphinicol pneumococcal isolates with MIC ≤ 8 µg/ml are susceptible and > 8 µg/ml are resistant. Trimethoprim sulphamethoxazole pneumococcal isolates with MIC ≤ 1 µg/ml are susceptible and > 2 µg/ml are resistant (50). All E-test products were from Biomērieux.

3.8.3. Quality-control procedures

Standard strain of *S. pneumoniae* ATCC 49619 and TIGR 4 were run in parallel to check the performance of the medium and potency of antibiotics. This quality control was done for every batch of media.

3.8.4 Serotyping

All culture positive pneumococcal isolates were tested by the Quellung reaction using serotyping sera from Staten Serum Institute (SSI), Copenhagen, Denmark. First pool antisera from A-I containing all serogroups and serotypes in one bottle were used followed by group and typing sera until the final serotype was determined. Serotyping test was performed by taking a 0.5µl platinum loopful of normal saline and suspending a pure colony in the saline with enough concentration. Then a loopful of antisera was added. The test was considered positive if swelling and clumping of cells was observed under the microscope. If the test was positive for one of the pool antisera, then testing again for groups and types included in that pool of antisera was further tested. When a type was obtained, testing was stopped but for groups factor sera were used to determine the type (11).

3.8.5. DNA preparation for MLST

100 µl of phosphate buffer saline (PBS) buffer was dispensed in an Eppendorf tube and 1 µl loop of pure colony was suspended in the buffer. After boiling at 100^{o}C in a dry bath for 10 minutes and centrifugation at 13000 rpm for 5 minutes, the supernatant was transferred to another clean Eppendorf tube. The tubes were stored at -20^{O} C until further testing.

Using the seven housekeeping genes primers listed in the table3 below, with a concentration of 10 µM the target genes were amplified followed by cleaning to get pure products. Then sequencing of the PCR product was performed using the same primers with a concentration of 1.6 µM. All procedures were performed using Eppendorf robot, automatically cleaning repeatedly followed by sequencing at Applied Biosystem sequencing machine. The primers of these genes are as follows for up and down strands.

Table 3 Primers used for MLST

Gene	Name	Primer name	Primer sequence
aroE	shikimate dehydrogenase	***aroE*-up** ***aroE*-dn**,	**5'-GCC TTT GAG GCG ACA GC** **5'-TGC AGT TCA (G/A)AA ACA T(A/T)T TCT AA**
gdh	glucose-6-phosphate dehydrogenase	***gdh*-up** ***gdh*-dn**	**5'-ATG GAC AAA CCA GC(G/A/T/C) AG(C/T) T** **5'-GCT TGA GGT CCC AT(G/A) CT(G/A/T/C) CC**
gki	glucose kinase	***gki*-up** ***gki*-dn**	**5'-GGC ATT GGA ATG GGA TCA CC** **5'-TCT CCC GCA GCT GAC AC**
recP	transketolase	***recP*-up** ***rec*P-dn**	**5'-GCC AAC TCA GGT CAT CCA GG** **5'- TGC AAC CGT AGC ATT GTA AC**
spi	signal peptidase I	***spi*-up** ***spi*-dn**	**5'-TTA TTC CTC CTG ATT CTG TC** **5'-GTG ATT GGC CAG AAG CGG AA**
xpt	xanthine phosphoribosyltransferase	***xpt*-up** ***xpt*-dn**	**5'-TTA TTA GAA GAG CGC ATC CT** **5'-AGA TCT GCC TCC TTA AAT AC**
ddl	D-alanine-D-alanine ligase	***ddl*-up** ***ddl*-dn**	**5'-TGC (C/T)CA AGT TCC TTA TGT GG** **5'-CAC TGG GT(G/A) AAA CC(A/T) GGC A**

Finally the resulting sequence type (ST) was interpreted by interring the result into the Bacterial Isolate Genome Sequence Database (BIGSdb) software for each of genes and sequence type identified for all pneumococcal isolates. (15, 48, 49)

3.8.6. Real- Time PCR (RT - PCR)

CSF samples which were negative by culture were tested using real time PCR (14). The CSF were cleaned by Qiagen cleaning solution QIAamp ® DNA mini kit, Qiagen staring 200 µl CSF finally 50 µl of ready to run clean sample and stored at -20^{o}C.

Table 4 RT PCR reagents and their proportion

Reagent	**Volume**	**Final concentration**
TaqMan Fast Universal PCR Master MIX	10 µl	1x
Nm, primer ctrA-F	0.6 µl	300 nM
primer ctrA-R	0.6 µl	300 nM
probe ctrA-P	0.8 µl	200 nM
Hi, primer omp P2-F	0.4 µl	200 nM
Primer omp P2-R	0.4 µl	200 nM
probe omp P2-P	0.8 µl	200 nM
Spn, primer F373	0.4µl	200 nM
Primer R424	0.4 µl	200 nM
probe pb400 Cy5	0.8 µl	200 nM
10x Exo IPC-mix	2 µl	1x
50x Exo IPCDNA	0.4 µl	1x
Nuclease free water	0.4 µl	

The cocktail was prepared according to the above proportion for one sample with final volume of reagent (n samples x n parallels) + 3. The reaction volume in this procedure was 20 µl. 2 µl clean product and 18 µl of cocktail. (Table 4) All samples were run with two parallels to avoid

false positives. Using multiple real time (RT)-PCR primers 140 CSF samples were tested for the presence of *N. meningitids, H. influenzae* and *S. pneumoniae* DNA. CSF collected using plain tube were tested directly and CSF collected using TI media were dilute 1:50 with Nuclease free water. Finally the reaction ran by a machine Applied bio system 7500 fast system. The reporter chromophor was FAM. (2) (Table 5). RT products with CI greater than 1 at both parallels considered as positive and positive only at a single parallel were re- assayed using three parallels to confirm. Annex 2

Table 5 Primers and probes sequence used for Real time PCR

Organism	**Primer**	**Primer sequence**	**Probe**
N.meningitidis	Ctr A F Ctr AR	5'TGTGTTCCGCTATACGCCATT GCCATATTCACACGATATACC	Ctr A-P 5' ACCTTGAGCAA"T"CCATTTA TCCTGACGTTCT
S.pneumoniae	Lyt A F373 Lyt A R424	5'ACGCAATCTAGCAGATGAAGCA 5' TCGTGCGTTTTAATTCCAGCT	Pb 400Cy5 5' FAM GCCGAAAACGC"T"TGATAC AGGGAG BHQ1
H.ifluenzae	Omp 2-F Omp 2 -R	5'GGTTAAATATGCCGATGGTGTTG 5' TGCATCTTTACGCACGGTGTA	Omp P2-P 5'TTGTGTACACTCCGT"T"GGT AAAAGAACTTGCAC

Table 6. Real time program

Procedure	Temprature	Time	Total
Denaturation	95^{o}C	20'sec	45 cycles
Annealing	60^{o}C	30' sec	
Extension	60^{o}C	30' sec	
Cooling	4^{o}C	∞	

3.9. Data management

Demographic and all microbiological, serological and molecular data were recorded electronically using Microsoft - Excel sheet to be retrieved by electronic copy in computer as well as with memory sticks for back up. The investigator was conducting periodic record audit at microbiology laboratories in order to check missed data comparing the hard copy of laboratory log books. The data were kept confidential for third party.

3.10. Statistical analysis

All recorded data were transferred from excel to SPSS version 20. From these used to prepare descriptive statistics and frequency tables and figures.

3.11. Ethical clearance

Ethical clearance was obtained from the Norwegian Ethical Review Board (REK) and the Scientific and Ethical Review Office at EHNRI. (Annex 3 and 4) Since samples were not collected for purpose of this study, consent or assent form was not mandatory. Instead for this study we have used the left over samples for the conformation of suspected causative agent.

4.12. Plan for the project

Table 7 Project timeline for the master's project

Activity	**Time**
Protocol writing	April 2012 - June 2012
Submiting the protocol to REK	June 2012
Ethical aproval from SERO/EHNRI	July 2012 - September 2012
Sample collection from the study sites and laboratory analysis /EHNRI	September 2012 - December 2012
Material transfer to NIPH	January 2013
Laboratory analysis /NIPH	February 2013 - April 2013
Data analysis and thesis write up	April 2013 - May 2013

Chapter four: Results

4.1 Patient characteristics

A total of 460 patients were included in the study. (Table 8) The gender ratio was 1:1 with 232 (50.5%) males and 228 (49.5%) females. The age distribution was from 4 month up to 51 years old with the mean age of 15. Of the 460 clinical samples, 265 were collected from pediatric patients under five years old; 228 (49.5%) were collected from invasive cases (sterile body sites) and 232 (50.5%) from non invasive (non sterile body sites) cases.

Table 8 Sample types and collection sites from Ethiopia

Sample type	Black Lion	Yek12	St. Paul	Addis Ketema	Woreda7	Gondar	Selam	EHNRI	Total
CSF	33	76	-	-	-	98	-	12	219
Blood	1	-	-	-	-	-	-	-	1
Throat swab	-	-	3	-	1	-	37	1	42
Sputum	-	-	98	39	25	-	14	2	179
Pleural fluid	1	-	8	-	-	-	-	-	9
Ear swab	-	-	4	-	-	-	-	6	10
Total	35	76	113	39	26	98	51	21	460

The CSF samples (219) predominated, followed by sputum (179), throat (42), ear (10) and pleural fluid (8). Only one blood sample was included in the study. More specimens were collected from St. Paul Hospital followed by Gondar University Hospital and Yekatit 12 Hospital.

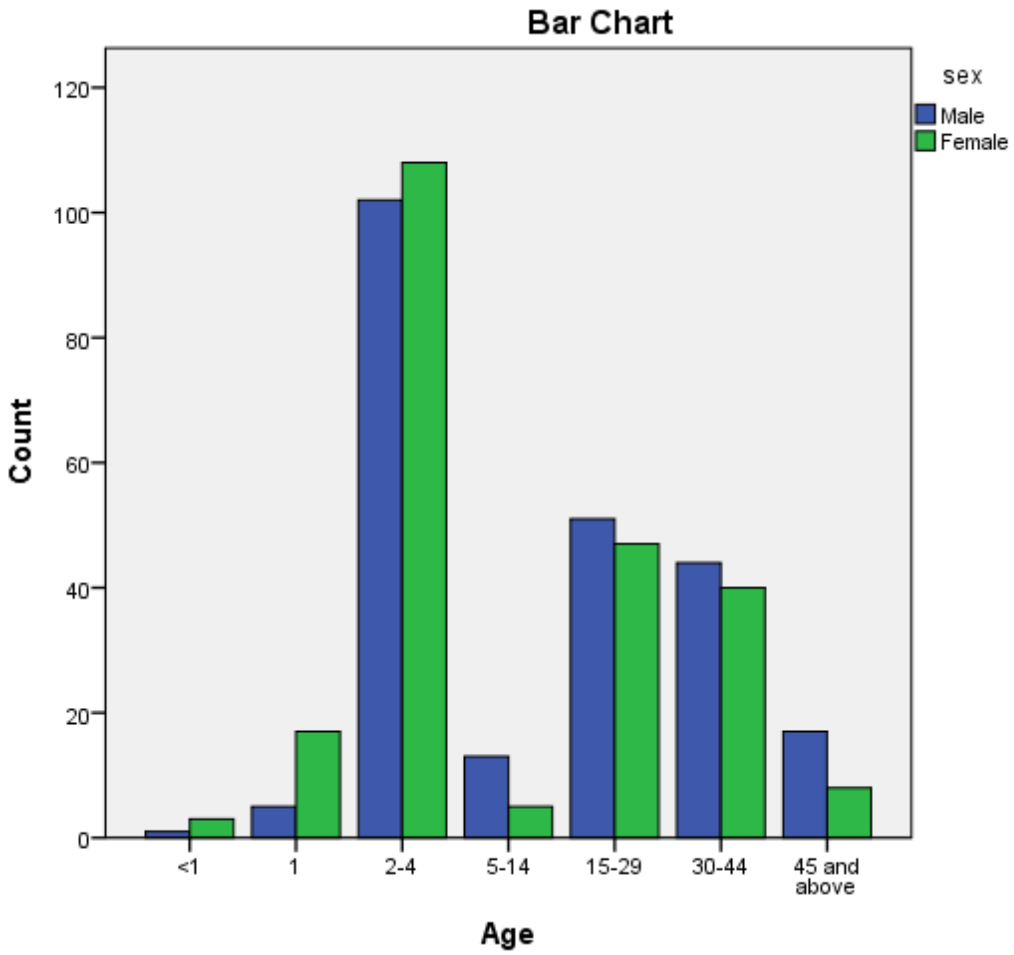


Figure 3 Bar chart age group of patients from Ethiopia with gender.

The ratio of male and female patients was similar in all age groups, except in the infant age group where the number of females outnumbered males. On the other hand in adult age groups the number of female outnumbered. (Figure 3)

4.2 Culture result

Table 9 Type of samples and their culture result

Sample	Culture				Total
	Negative	Percent	Positive	Percent	
Sputum	172	96%	7	4%	179
Throat	25	59%	17	41%	42
CSF	196	89%	23	11%	219
Pleural	5	56%	4	44%	9
Blood	0		1		1
Ear	1	10%	9	90%	10
Total	399		61		460

Out of the 460 samples, 61 were culture positive (Table 9). The pneumococcal culture positive rate was 61/ 460 (13%). These included 28 (45%) samples from invasive diseases and 33 (55%) from non invasive diseases. The highest number of pneumococcal culture positive was observed in CSF samples, followed by throat samples and ear samples. According to the proportion of samples, ear swab, pleural fluid and throat samples have higher positive rate of pneumococcus 90%, 44% and 41%, respectively.

Table 10 Culture result according to age

Age group	Culture		Total
	Positive	Negative	
<1	0	4	4
1	0	22	22
2-4	28	181	209
5-14	15	3	18
15-29	6	92	98
30-44	6	78	84
45 and above	7	18	25
Total	61	399	460

The culture positive rate was higher in children less than 14 years of age, 43 /61 (70%) pneumococcal isolates were from this age group. Most of the pneumococcal isolates due to invasive cases (isolates from sterile sites) were from this age group 24/28 (86%). In this study infant age groups (<1 years of age) have no pneumococcal findings (Table 10).

4.3 Serotype result

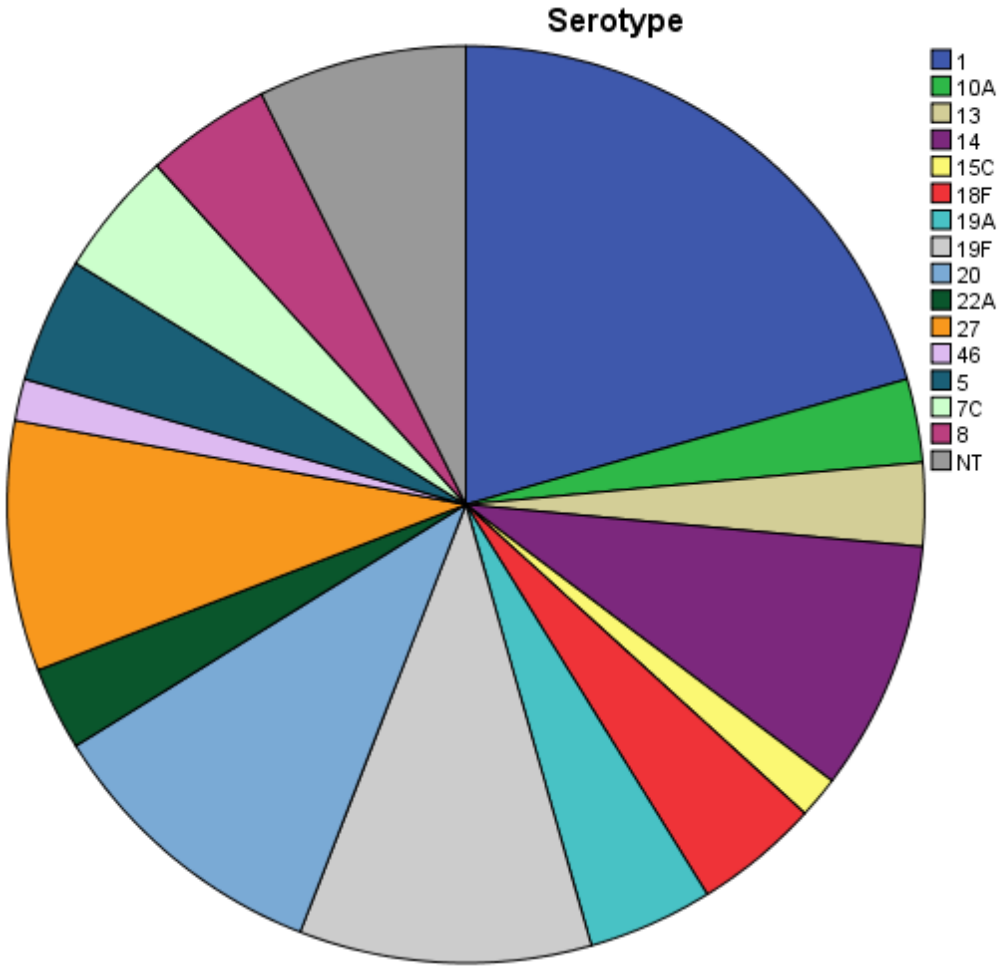


Figure 4 Pneumococcal serotype distribution from patients in Ethiopia

Of the 61 samples positive for pneumococci in culture, 6 samples from non invasive diseases harbored multiple serotypes : these were 4 throat samples, 1 sputum and 1 ear swab. In addition, one 19F and one NT strain were recovered from one CSF sample. (Table 11) The total number of isolates became 68.

Table 11 Multiple serotypes and their sources

Multiple serotypes	**Source**	**STs**
1 + NT	Throat	1(217) & NT(2711)
27 + NT	Throat	27(1475) & NT (1475)
27 + NT	Throat	27(1475) & NT(1475)
14 + 18F	Sputum	14 (63) &
19F + NT	CSF	19F(1203) &NT(1203)
19F + NT	Ear	19F(1203) &NT(1203)
20 + 1	Throat	20(6451) & 1(303)

In this study 16 serotypes were identified and serotype 1 was the leading cause of pneumococcal infection, followed by serotypes 19F and 20. Serotypes 14 and 27, non typable serotypes, 19A, 18F, 5, 7C and 8 and 10, 13, and 22A, and 15, and 46 have their own contribution. All serotypes were isolated from CSF except 18F, 7C, 13, 15C and 46 which were only isolated from non invasive (non sterile) sites. Serotype 1 was isolated from all types of clinical samples, except ear swab. (Table 12)

Table 12 serotype distribution according to site of isolation

Serotype	Frequency	Site of isolation					
		CSF	Throat	Ear	Sputum	Pleural	Blood
1	14	5	4	0	2	2	1
19F	7	3	2	2	0	0	0
20	7	3	1	3	0	0	0
14	6	3	0	0	3	0	0
27	6	1	3	0	0	2	0
NT	5	1	3	1	0	0	0
19A	3	2	1	0	0	0	0
18F	3	0	2	0	1	0	0
5	3	1	1	1	0	0	0
7C	3	0	1	0	1	0	0
8	3	1	1	1	0	0	0
10A	2	2	0	0	0	0	0
13	2	0	1	0	1	0	0
22A	2	1	0	0	0	0	0
15C	1	0	0	1	0	0	0
46	1	0	0	1	0	0	0
Total	68	23	20	10	8	4	1

4.4 Antimicrobial susceptibility

Table 13. MIC results of the 68 pneumococcal isolates

Antibiotics	**MIC in µg/ml**											
	0.0 16	**0.032**	**0.064**	**0.125**	**0.250**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**
Penicllin G	21	23		5	12	4	3					
Ceftriaxone	37	12	4	5	6	3					1	
Erythromycin		4	10	45				3	4	1	1	
Tetracycline			2	42	6			1		2	7	8
Chloramphenicol								37	28			3
Trimethoprim-sulphamethoxazole				3	17	23		4	7	3	4	7

The susceptibility pattern of pneumococcal isolates were interpreted from Table 13.

Table 14 Antibiotics and their result

Antibiotics	**Susceptible**	**Intermediate**	**Resistant**
Penicillin	44	24	0
Ceftriaxone	67	0	1
Erythromycin	59	0	9
Tetracycline	50	1	17
Chloramphenicol	65	0	3
Trimethoprim-sulphamethoxazole	43	4	21

Of the 68 pneumococcal isolates 44 were susceptible for penicilin and 24 were intermediate resistant. Thirteen CSF isolates were susceptible for penicillin and the remaining were intermediate resistant. All but one pneumococcal isolates were susceptible to ceftriaxone; the resistant isolate was from ear swab. Nine isolates were resistant to erythromycin; those were 3 from CSF, 3 from throat, 1 from blood, 1 from ear and 1 from pleural fluid. Fifty pneumococcal isolates were susceptible to tetracycline, 1 was intermediate resistant and 17 were resistant (9 from CSF, 1 pleural, 1 blood and the remaining ones from non invasive sites). Three isolates were resistant to chloramphenicol, 1 from CSF, 2 from throat samples. Four isolates were intermediate resistant to trimethoprim - sulphamethoxazole and 21 resistant (11 from CSF, 1 from pleural fluid and the remaining ones were from non invasive (Table 14) . In this study there is no significance difference in susceptibility pattern between pneumococcal isolates of invasive and non invasive diseases.

4.5. Multilocus sequence typing results

Table 15 Result of sequence typing for 68 pneumococcal isolates from Ethiopia

Sequence type (ST)	Frequency	Serotypes
63	6	14
217	8	1
289	3	5
303	2	1
1203	9	19 F = 7 and NT=2
1475	8	27 = 6 and NT= 2
2054	3	7C
2345	3	19A
2711	4	18F = 3 and NT = 1
6451	7	20
8873	2	22A
8874	3	8
8875	2	13
8876	2	10A
8877	4	1
8974	1	15C
8975	1	46

All the data from the seven housekeeping genes were entered into the software and analyzed. After summing up all results of the seven housekeeping genes 17 STs were identified, of which 7 were new (8873, 8874, 8875, 8876, 8877, 8974 and 8975). Out of the seven multiple serotypes four of them were the same clone, three of them were different serotypes and different clones. (Table 15).

Table 16 ST associated with source of sample from patients in Ethiopia

ST	Sample					
	CSF	**Throat**	**Ear**	**Sputum**	**Pleural**	**Blood**
63	3	0	0	3	0	0
217	2	2	0	2	2	0
289	1	1	1	0	0	0
303	1	1	0	0	0	0
1203	3	2	2	0	0	0
1475	1	5	0	0	2	0
2054	0	2	0	1	0	0
2345	2	1	0	0	0	0
2711	0	2	0	1	0	0
6451	3	1	3	0	0	0
8873	2	0	0	0	0	0
8874	1	1	1	0	0	0
8875	0	1	0	1	0	0
8876	2	0	0	0	0	0
8877	2	1	0	0	0	1
8974	0	0	1	0	0	0
8975	0	0	1	0	0	0

Some STs are isolated from multiple samples as well as invasive and non invasive samples. Fifteen pneumococcal isolates were from the new alleles and 8 from invasive diseases and 7 of them were from non invasive. Out of the new STs 2 of them were only from invasive cases, 3 from non invasive and 2 from both sites.

4.6 Vaccine coverage

Out of serotypes identified by this study 4 serotypes of PCV-10 and 5 serotypes of PCV-13 are found.

Table 17 Vaccine coverage of serotypes from Ethiopia

Vaccine	**Coverage total (n=68)**	**Coverage invasive (n=28)**
PCV-10(**1.5,14,19F**)	44%	45%
PCV-13(**1.5,14,19F,19A)**	49%	52%

The main target of vaccine is for the prevention of invasive diseases and if we consider invasive isolates independently, the vaccine coverage will be 45% and 52% for PCV-10 and PCV13 respectively. non vaccine serotypes

4.7 Real Time PCR results

Table 18 Result of 140 culture negative CSF from Ethiopia

Organism	**Frequency**
S. pneumoniae	9
N. meningitidis	12
S. pneumoniae + N. meningitidis	1
S. pneumoniae + H. influenzae	1
Negative	117
Total	140

In this study 140 culture negative CSF were run by RT-PCR and 23 of them were positive for meningitis agents. *N. meningitidis* were the leading agent (n=12) followed by *S. pneumoniae (n=9)* and two samples were positive for multiple agents one with *S. pneumoniae + N. meningitidis* and *S. pneumoniae + H. influenzae.* (Table 18)

Table 19 RT-PCR result according to study sites

Study site	**Isolate**				
	Spn	*Nm*	*Hi*	*Nm + Spn*	*Spn + Hi*
Gondar University Hospital	6	10	0	1	1
Black Lion Hospital	3	1	0	0	0
Yekatit 12 Hospital	0	1	0	0	0

Most of RT-PCR positive results (n=18) were from Gondar University Hospital followed by Black Lion Hospial and 1 from Yekatit 12 Hospital. Mixed results were also from the more positive study site; it might be due to contamination at the laboratory or sample collection.

Chapter 5 Discussion

5. 1 Sample collection

Pneumococcus has a great public health importance worldwide due to its medical impact up on the society. The main burden of the bacterium are its multiple infections followed by high mortality rate, complications after cure, resistance to empirical antibiotics, as well as variation among strains circulating in a given community. The fatality and prevalence of the organism is higher relative to other similar agents for a specific disease (51). It is vaccine preventable disease and currently different types of vaccines that have great impact for the reduction of its burden are produced. Since the vaccine is serotype specific, prior to or the introduction of vaccine, information about pneumococcal serotypes circulating in the community is mandatory. Otherwise the effect of the vaccine will be difficult to evaluate (52).

The aim of this study was to characterize pneumococcal isolates circulating in Ethiopia by taking clinical samples from patients including all age groups. A total of 460 clinical samples were collected from 228 invasive (sterile body sites) and 232 non invasive cases almost with 1:1 ratio. Including both types of pneumococcal cases will have good advantage identifying carriers and diseased individuals as well as identifying serotypes that can cause fatal cases. A study conducted in Niger for the determination of pneumococcal serotypes, a total of 19, 223 CSF were collected all over the country, in 8 years surveillance.(53) The time available for this study (6 months) prevented a more extensive and focused study.

5.2 Culture results

The general culture positive rate in this study was around 13% (61/460). Specifically for each type of samples, CSF had around 11% (23/219) but other studies suggested that CSF culture among suspected meningitis case, the positive rate would be around 16% (54)but in this study the rate was very low. These samples were negative for other meningitis causing agents. The culture detection rate was low and it might be due to other confounding factors (prior antibiotic treatment, other meningitis agents, clinical criterias to diagnose for bacterial meningitis). In this study all samples were left over samples and we have no information about these factors. Most of CSF samples were clear indicating that non pyogenic meningitis and all meningitis agents other than bacteria were not addressed in this study. Meningitis besides bacterial agents, can also be caused by viruses, parasites, and fungal elements (55). This might have its own contribution

for low detection rate. The rate of pneumococcal isolation is seasonal and collecting samples beyond the pick season also may reduce its yield. All infant patients up the age of 12 months(1 year) were culture negative. This might be in part due to the introduction of PCV-10 vaccine in Ethiopia a year before. A study conducted in Israel similar to this study that after the introduction of 9- valent vaccine, the carriage rate was reduced after the introduction of the vaccine (56). The pneumococcal cases (55%) were mainly between the age of 1 and 5 who are the vulnerable group for the agent.

5.3 Antimicrobial susceptibility test results

Penicillin G is the primary choice for the empirical treatment of pneumococcal infections but in this study 35% (24/68) of the isolates were intermediate resistant for penicillin, but none were resistant. For other bactericidal antibiotics (ceftriaxone and chloramphenicol) all pneumococcal isolates were susceptible except one resistant for ceftriaxone and three for chloramphenicol. Bacteriostatic antibiotics erythromycine, tetracycline and trimethoprim-sulphametoxazole had resistance rate of 13% (9/68), 26.5% (18/68) and 37% 25/68 respectively. From meningitis cases 46% (11/24) were resistant to penicillin while the 5 other invasive isolates (pleural and blood) were susceptible. Non invasive isolates had a higher resistance rate, especially throat followed by sputum isolates. A study conducted in Gondar Ethiopia, nasopharyngeal carriage and antimicrobial susceptibility pattern, the susceptibility pattern of isolates is similar except more penicillin resistant isolates in this study (57). Studies conducted in West Africa, the Gambia and Kenya show that there is significance increment of resistance rate in bactericidal antibiotics (17, 18).

5.4 Serotype results

Identifying pneumococcal isolates either at invasive or non invasive cases is important determining serotypes circulating in the community for preventive and epidemiological aspects. In this study, from both invasive and non invasive cases a total of 16 serotypes were identified including non typable. Serotype 1 was the leading cause of pneumococcal infection followed by 19F and 20 serotypes among Ethiopian patients. This study agrees with similar studies conducted in the Gambia, West Africa and Kenya, East Africa showing that serotype 1 is the

leading cause of pneumococcal infection in Africa (18, 58). Non typable isolates were identified from non invasive clinical samples. One non typable isolate was identified from CSF together with 19F isolate. Both isolates had the same ST and it might be that the organism could lose its capsule and become non typable (58). But other serotypes most common in the above studies(6A, 6B, 23F) were not encountered by this study. Out of the PCV-10 serotypes only 4 of them (1, 5, 14 and 19F) were included in this study whereas 5 (1, 5, 14, 19F and 19A) serotypes of PCV 13 were also included in this study. Considering invasive pneumococcal isolates, the vaccine coverage for PCV-10 and PCV-13 would be 45% and 52% respectively. Unless there is cross reaction between group serotypes the current PCV vaccines coverage is minimal for serotypes identified in this study.

5.5 MLST results

MLST is used to identify the similarities between isolates using housekeeping genes which are common for all clinical isolates. The data is used to classify pneumococcal isolates according to the degree of relation among serotypes for molecular epidemiology. In this study 17 STs were identified among the 61 isolates and out of these 7 STs were identified in this study. It is a significant contribution of new data on STs from a country with no previous data.

5.6 CSF RT-PCR results

RT-PCR has a high sensitivity and specificity identifying bacterial pathogens from clinical samples. The method has high sensitivity detecting up to a single DNA molecule (2). In this study 140 culture negative CSF were tested for the presence of bacterial pathogens, *N. meningitidis*, *H. influenzae* and *S. pneumoniae* (14, 59). Out of 140 culture negative CSF samples, 23 were positive for meningitis associated agents and two of them were mixed agents. Identifying mixed organisms from the very sterile sites is rare and it might be due to contamination during sample collection or laboratory analysis since these mixed organisms from the same study sites. Care should be taken to assure good quality of samples for such sensitive analysis as RT-PCR.

5.6 Conclusion

In this study 15 serotypes of *S. pneumoniae* were identified and only four and five of them were included in the current vaccines PCV-10 and PCV-13, respectively. The remaining serotypes were from non vaccine groups. The current vaccine in Ethiopia is PCV-10 and if this study was considered as background for the current vaccine, 44% coverage would be expected which is below the minimum desirable coverage.

5.7 Recommendation

A good vaccine should cover of more than 80% of the strains circulating in the community. Due to this the vaccine already introduced in the immunization program should be validated with current findings on pneumococcal serotypes. Even according to this study the PCV-10 vaccine coverage is not good enough to be continued in the immunization program.

Another enhanced pneumococcal surveillance should be performed with large number of samples using this study as a background.

Susceptibility of isolates to antimicrobials which are prescribed in the health facilities should be checked in microbiology laboratory prior to empirical treatment.

Awareness should be created among community members in order to avoid self prescription of antibiotics, especially oral administered ones.

Quality control should be routine part of the laboratory.

All events in the laboratory should be recorded to trace unexpected through daily practices.

Refreshment training is mandatory to have current practices about the subject matter globally.

Sensitive laboratory methods should be part of diagnosis at least at the referral level.

Better to introduce good laboratory practice at any procedure, to prevent contaminations incan prevent

5.8 Strong sides of the study.

- Good quality control measures were followed throughout the study and many laboratory parameters were included which can be supplemental for each other.

- Current and validated laboratory procedures were followed in well equipped and accredited laboratory.

- Results were compared by senior laboratory personnel.

5.9 Limitations of the study.

- Duration of the study is short in order to include seasonal incidence of pneumococcal infection.

- Seasonal variation of circulating serotypes were not identified.

- Even though it is better relative to previous studies in Ethiopia still the number of isolates are not good enough to generalize for the country.

- Study sites are so small relative to the total population of the country.

Chapter six Reference

1. O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, Lee E, Mulholland K, Levine OS, Cherian T; Hib and pneumococcal global burden of disease study team. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet, 2009; 374:893-902.

2. World Health Organization. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*. 2011; 2nd Edition, 73-90, 105-152.

3. Levinson W. Review of Medical Microbiology and Immunology. 12th Edition, 2006 (Lange Medical Books).

4. Ortqvist A, Hedlund J, Kalin M. *Streptococcus pneumoniae*: epidemiology, risk factors, and clinical features. Semin Respir Crit Care Med, 2005; 26:563-74.

5. Pneumococcal disease, http://www.cdc.gov/vaccines/pubs/pinkbook/downloads/pneumo.pdf, (Accessed date 24/03/2013).

6. Facklam R. What happened to the streptococci: overview of taxonomic and nomenclature changes. Clin Microbiol Rev, 2002;15:613-630. Review.

7. Harvey R. A., Champe P. C. and Fisher B.D. Lippincott's Illustrated Reviews: Microbiology. 2nd Edition, 2007.

8. Thompson CC, Emmel VE, Fonseca EL *et al.* (2013) Streptococcal taxonomy based on genome sequence analyses. [v1; ref status: indexed, http://f1000r.es/o1] *F1000Research* ,

2013; **2**:67.

9. Cheesbrough M. District laboratory practice in tropical countries. Part two, 2006; 2nd Edition. Cambridge University Press, UK.

10. Todar,K online text book of Microbiology *Streptococcus pneumoniae* and *Streptococcal* Disease. http://textbookofbacteriology.net/S.pneumoniae.html (Accessed 04/10/2012).

11. Staten Serum Institut, Manufacturer's leaflet. 2001.

12. **Jorgensen** JH, Ferraro MJ. Antimicrobial Susceptibility Testing: A review of general principles and contemporary practices. Clin Infect Dis. 2009; 49:1749-1755.

13. Yaro S, Lourd M, Traoré Y, Njanpop-Lafourcade BM, Sawadogo A, Sangare L, Hien A, Ouedraogo MS, Sanou O, Parent du Châtelet I, Koeck JL, Gessner BD. Epidemiological and molecular characterstics of a highly lethal pneumococcal meningitis epidemic in Burkina Faso. Clin Infect Dis, 2006; 43:693-700.

14. Carvalho Mda G, Tondella ML, McCaustland K, Weidlich L, McGee L, Mayer LW, Steigerwalt A, Whaley M, Facklam RR, Fields B, Carlone G, Ades EW, Dagan R, Sampson JS. Evaluation and improvement or real-time PCR assays targeting lytA, ply, and psaA genes for detection of pneumococcal DNA. J Clin Microbiol, 2007; 45:2460-2466.

15. **Maiden** MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feavers IM, Achtman M, Spratt BG. Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A, 1998; 95: 3140-3145.

16. Melanie Abeyta, Gail G. Hardy, and Yother J. Genetic alteration of capsule type but not PspA type affects accessibility of surface-bound complement and surface antigens of *Streptococcus pneumoniae*. Infect. Immun, 2003; 71:218– 225.

17. **Scott** JA, Hall AJ, Hannington A, Edwards R, Mwarumba S, Lowe B, Griffiths D, Crook D,

Marsh K. Serotype distribution and prevalence of resistance to benzylpenicillin in three representative populations of *Streptococcus pneumoniae* isolates from the coast of Kenya. Clin Infect Dis, 1998 Dec;27(6):1442-50.

18. **Adegbola** RA, Hill PC, Secka O, Ikumapayi UN, Lahai G, Greenwood BM, Corrah T. Serotype and antimicrobial susceptibility patterns of isolates of *Streptococcus pneumoniae* causing invasive disease in The Gambia 1996–2003. Trop Med Int Health, 2006;11:1128-1135.
19. **Mudhune** S, Wamae M; Network Surveillance for Pneumococcal Disease in the East African Region. Report on invasive disease and meningitis due to *Haemophilus influenzae* and *Streptococcus pneumoniae* from theNetwork for Surveillance of pneumococcal Disease in the East African Region. Clin Infect Dis, 2009;48 Suppl 2:S147-152.
20. **Lynch** JP 3rd, Zhanel GG. *Streptococcus pneumoniae*: epidemiology and risk factors, evolution of antimicrobial resistance, and impact of vaccines. Curr Opin Pulm Med, 2010; 16:217-25.
21. **Giebink** GS. The prevention of pneumococcal disease in children. N Engl J Med, 2001; 345(16):1177-83
22. Adamou JE, Wizmann TM, Barren P, Langermann S. Adherence of *Streptococcus pneumoniae* to human bronchial epithelial cells (BEAS-2B). Infect Immun, 1998;66:820-822.
23. **Lynch** JP 3rd, Zhanel GG. *Streptococcus pneumoniae*: epidemiology, risk factors, and

strategies for prevention. Semin Respir Crit Care Med, 2009;30:189-209.

24. **Mitchell** AM, **Mitchell** TJ. *Streptococcus pneumoniae*: virulence factors and variation. Clin Microbiol Infect, 2010; 16:411-418

25. **Gillespie** SH, Balakrishnan I. Pathogenesis of pneumococcal infection. J Med Microbiol, 2000; 49:1057-1067.

26. **Nelson** AL, Roche AM, Gould JM, Chim K, Ratner AJ, Weiser JN. Capsule enhances pneumococcal colonization by limiting mucus mediated clearance. Infect Immun, 2007; 75:83-90.

27. **King** SJ, Hippe KR, Gould JM, Bae D, Peterson S, Cline RT, Fasching C, Janoff EN, Weiser JN. Phase variable desialylation of host proteins that bind to *Streptococcus pneumoniae* in vivo and protect the air way. Mol Microbiol, 2004; 54:159-71.

28. **Barocchi** MA, Ries J, Zogaj X, Hemsley C, Albiger B, Kanth A, Dahlberg S, Fernebro J, Moschioni M, Masignani V, Hultenby K, Taddei AR, Beiter K, Wartha F, von Euler A, Covacci A, Holden DW, Normark S, Rappuoli R, Henriques-Normark B. A pneumococcal pilus influences virulence and host inflammatory responses. Proc Natl Acad Sci U S A, 2006; 103:2857-62.

29. **van der Poll** T, Opal SM.Pathogenesis, treatment, and prevention of pneumococcal pneumonia. Lancet, 2009; 374:1543-1556.

30. **Jacobs** MR, Koornhof HJ, Robins-Browne RM, Stevenson CM, Vermaak ZA, Freiman I, Miller GB, Witcomb MA, Isaäcson M, Ward JI, Austrian R. Emergence of multiply resistant pneumococci. N Engl J Med, 1978; 299:735-740.

31. Forward KR. The epidemiology of penicillin resistance in *Streptococcus pneumoniae*. Semin Respir Infect, **1999** ;14:243-254. Review.

32. http://www.who.int/drugresistance/technicalguidance/en/resistantinfection.pdf (Accessed 17/4/2013

33. **Porco** TC, Gebre T, Ayele B, House J, Keenan J, Zhou Z, Hong KC, Stoller N, Ray KJ, Emerson P, Gaynor BD, Lietman TM. Effect of mass distribution of azithromycin for trachoma control on overall mortality in Ethiopian children: a randomized trial. JAMA, 2009;302:962-968.

34. Martín-Galiano AJ, de la Campa AG. High-efficiency generation of antibiotic –resistant strains of *Streptococcus pneumoniae* by PCR and transformation. Antimicrob **Agents** Chemother, 2003;47:1257-1261.

35. **Muhe** L, Klugman KP. Pneumococcal and *Haemophilus influenzae* meningitis in a children's hospital in Ethiopia: serotypes and susceptibility patterns. Trop Med Int Health, 1999; 4:421-427.

36. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Twenty second informational supplement, 2012; M100-S22(27).

37. **Briles** DE, Tart RC, Swiatlo E, Dillard JP, Smith P, Benton KA, Ralph BA, Brooks-Walter A, Crain MJ, Hollingshead SK, McDaniel LS.Pneumococcal diversity: considerations for new vaccine strategies with emphasis on pneumococcal surface protein A (PspA). Clin Microbiol Rev, 1998;11:645-657.

38. Pneumococcal Vaccines (PCV13 and PPSV23)
http://www.immunize.org/askexperts/experts_pneumococcal_vaccines.asp.
(Accessed 3/2/2013).

39. http://www.immunisation.ie/en/Downloads/NIACGuidelines/PDFFile_15486_en.pdf;

40. http://www.who.int/vaccines/en/pneumococcus.shtml (Accessed 25/03/2013)

41. Central Statistical agency and ORC Macro. Ethiopia Demographic and Health Survey 2005. Addis Ababa and Calverton, MD: Central Statistical Agency and ORC Macro 2006.

42. Health and Health Related Indicators. Federal Ministry of Health. Ethiopia, 2010.

43. http://www.who.int/csr/disease/meningococcal/BulletinMeningite2012_S07.pdf (Accessed 15/4/2013)

44. World Health Organization. Weekly epidemiological record . 2011; 80:313-320

45. Hoffmanbros. The last exodus of Ethiopian Jews. Little History. May 08, 2011. http://www.kisskissbankbank.com/le-dernier-exode-des-juifs-d-ethiopie
(Accessed 16/5/2013)

46. Microbiology Specimen Collection and Transport, http://www.healthcare.uiowa.edu/path_handbook/Appendix/Micro/micro_spec_collection.html
(Accessed 09/07/2012)

47. Sample collection procedure manual, Diagnostic services of Manitoba Inc. Microbiology. http://www.dsmanitoba.ca/professionals/files/Policy_120-10-05.pdf (Accessed 16/09/2012)

48. **Meats** E, Feil EJ, Stringer S, Cody AJ, Goldstein R, Kroll JS, Popovic T, Spratt BG.

Characterization of encapsulated and non capsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. J Clin Microbiol, 2003; 41:1623-1636.

49. http://spneumoniae.mlst.net/misc/info.asp. pneumococcal MLST database which is located at Imperial College London and is funded by the Wellcome Trust'. (Accessed Feb. 2 2013)

50. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 3.1, 2013. http://www.eucast.org

51. **Ramakrishnan** M, Ulland AJ, Steinhardt LC, Moïsi JC, Were F, Levine OS. Sequelae due to bacterial meningitis among African children: a systematic literature review. BMC Med, 2009; 7:47.

52. www.preventpenumo.org

53. **Collard** JM, Alio Sanda AK, Jusot JF. Determination of pneumococcal serotypes in meningitis cases in Niger, 2003- 2011.PLoS One, 2013;8(3):

54. **Chanteau** S, Sidikou F, Djibo S, Moussa A, Mindadou H, Boisier P. Scaling up of PCR-based surveillance of bacterial meningitis in the African meningitis belt: indisputable benefits of multiplex PCR assay in Niger. Trans R Soc Trop Med Hyg. 2006; 100:677-680.

55. Meningitis. CDC. http://www.cdc.gov/meningitis/index.html , (Accessed 06/05/2013)

56**. Dagan** R, Givon-Lavi N, Zamir O, Sikuler-Cohen M, Guy L, Janco J, Yagupsky P, Fraser D. Reduction of nasopharyngeal carriage of *Streptococcus pneumoniae* after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. J Infect Dis, 2002 ;185:927-936.

57. **Assefa A**, Gelaw B, Shiferaw Y, Tigabu Z. Nasopharyngeal carriage and antimicrobial susceptibility pattern of *Streptococcus pneumoniae* among pediatric outpatients at Gondar

University Hospital, North West Ethiopia. Pediatr Neonatol. 2013

58. **Kisakye** A, Makumbi I, Nansera D, Lewis R, Braka F, Wobudeya E, Chaplain D, Nalumansi E, Mbabazi W, Gessner BD.Surveillance for *Streptococcus pneumoniae* meningitis in children aged <5 years: implications for immunization in Uganda. Clin Infect Dis, 2009; 48 Suppl 2:S153-S161.

59. Chiba N, Murayama SY, Morozumi M, Nakayama E, Okada T, Iwata S, Sunakawa K, Ubukata K. Rapid detection of eight causative pathogens for the diagnosis of bacterial meningitis by real-time PCR. J Infect Chemother, 2009; 15:92-98.

60. Vestrheim DF, Høiby EA, Aaberge IS, Caugant DA. Phenotypic and genotypic characterization of *Streptococcus pneumoniae* strains colonizing children attending day-care centers in Norway. J Clin Microbiol, 2008; 46:2508-2518

61. Lynch JP 3rd, Zhanel GG. *Streptococcus pneumoniae*: does antimicrobial resistance matter? Semin Respir Crit Care Med 2009; **30**:210–238. In depth review of escalation of antimicrobial resistance globally among pneumococci, and the intercontinental spread of a few MDR clones.

Annex 1 General information of pneumococcal isolates from Ethiopia

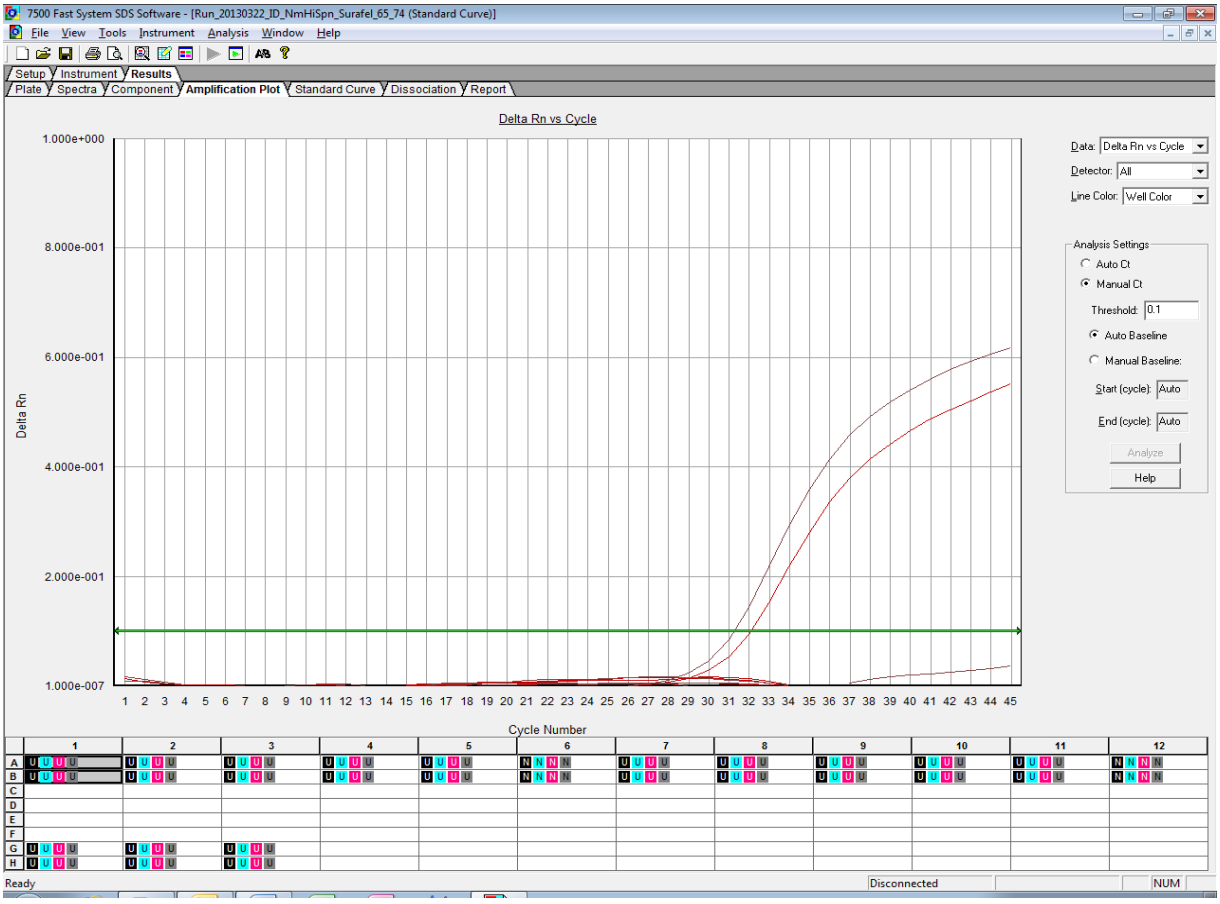
Supplement Table

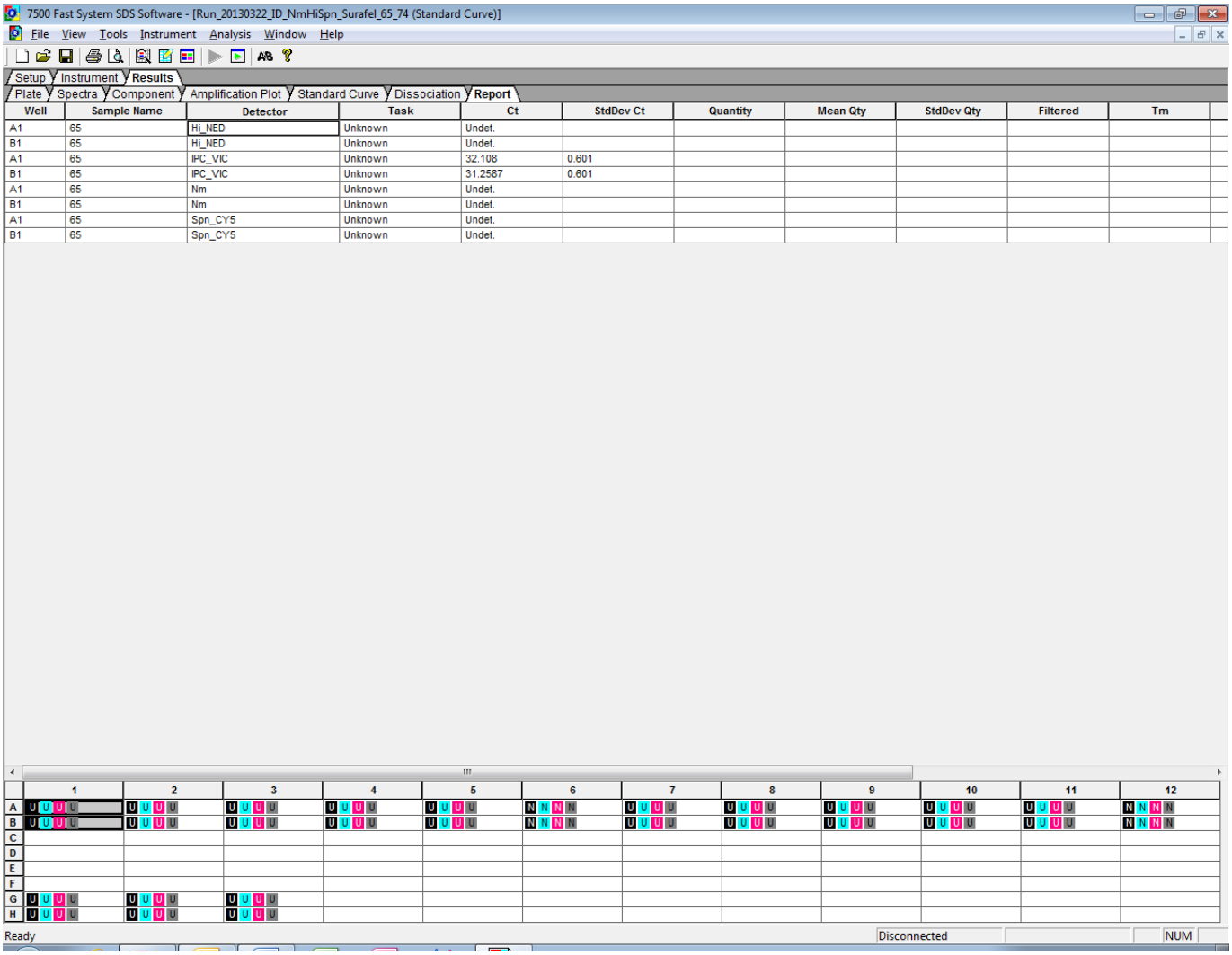
Isolate	Clinical source	Study site	Age	Sex	Serogroup	Sequence type
1	Ear	EHNRI	6	Female	20	6451
2	CSF	EHNRI	4	Male	5	289
3	Ear	EHNRI	7	Male	20	6451
4	CSF	EHNRI	5	Male	14	63
5	CSF	EHNRI	4	Female	1	217
6	Ear	EHNRI	3	Male	8	8874
7	Throat	EHNRI	16	Male	19F	1203
8	Ear	EHNRI	7	Male	20	6451
9	CSF	EHNRI	4	Female	14	63
10	CSF	EHNRI	6	Female	14	63
11	CSF	EHNRI	3	Female	20	6451
12	CSF	EHNRI	5	Female	20	6451
13	CSF	EHNRI	7	Male	20	6451
14	CSF	EHNRI	5	Male	1	303
15	CSF	EHNRI	4	Male	22A	8873
16	CSF	EHNRI	6	Male	22A	8873
17	CSF	EHNRI	4	Female	19F	1203
18	Sputum	Addis Ketema	29	Female	14	63
19	Sputum	Addis ketema	38	Female	1	217
20	Throat	Wereda 7	32	Female	5	289
21	Sputum	St. Paul	32	Female	13	8875
22	Ear	St Paul	12	Male	5	289
23	Ear	St Paul	11	Male	19F	1203
24	Ear	St Paul	13	Male	19F	1203
25	Throat	St Paul	16	Male	13	8875
26	Throat	St Paul	15	Male	18F	2711
27	Sputum	St Paul	45	Male	18F	2711
28	Sputum	St Paul	35	Male	14	63
29	Pleural fluid	St Paul	31	Male	27	1475
30	Sputum	St Paul	46	Female	1	217
31	Throat	Sealm Health Center	2	Male	1	217
32	Pleural	Black Lion	21	Male	1	217
33	Throat	Sealm	2	M	27	1475
34	Throat	Sealm	3.2	M	1	303
35	Throat	Sealm	3	Female	18F	2711

36	Throat	Sealm	2.8	Male	19F	1203
37	Throat	Sealm	4	Male	27	1475
38	Throat	Sealm	3	Female	8	8874
39	Throat	Sealm	4.6	Female	27	1475
40	CSF	Black Lion	4	Female	27	1475
41	CSF	Black Lion	3	Male	8	8874
42	Pleural	St. Paul	34	Male	27	1475
43	Sputum	St.Paul	14	Male	7C	2054
44	Pleural	St. Paul	21	Female	1	217
45	Throat	Selam	2.5	Female	7C	2054
46	Throat	Sealm	3.6	Female	19A	2345
47	Throat	Selam	4.8	Male	7C	2054
48	Throat	Selam	5	Feamle	1	217
49	Throat	Sealm	1.9	Male	1	8877
50	CSF	Gondar	4	Male	10A	8876
51	CSF	Gondar	3	Female	10A	8876
52	CSF	Gondar	4	Male	1	8877
53	CSF	Gonadr	1.5	Female	1	217
54	CSF	Gondar	2	Female	19F	1203
55	CSF	Gondar	3	Female	19F	1203
56	CSF	Black Lion	4	Female	19A	2345
57	CSF	Black Lion	3	Female	19A	2345
58	Blood	Black Lion	5	Female	1	8877
59	CSF	Black Lion	3.5	Male	1	8877
60	Ear	EHNRI	5	Male	46	8975
61	Ear	EHNRI	3	Male	15C	8974
62	CSF	EHNRI	4	Female	Non Typable	1203
63	Sputum	St.Paul	45	Male	14	63
64	Ear	St.Paul	11	Male	Non-Typable	1203
65	Throat	Selam	3.2	M	20	6451
66	Throat	Selam	2	M	Non-Typable	1475
67	Throat	Selam	4	M	Non-Typable	1475
68	Throat	Selam	2	M	Non-Typable	2711

Annex 2 Sample of Real Time PCR assay

Run_20130322_ID_NmHiSpn_Surafel_65_74_results




7500 Fast System SDS Software - [Run_20130322_ID_NmHiSpn_Surafel_65_74 (Standard Curve)]
File View Tools Instrument Analysis Window Help
Setup Instrument Results
Plate Spectra Component Amplification Plot Standard Curve Dissociation Report
Well Sample Name Detector Task Ct StdDev Ct Quantity Mean Qty StdDev Qty Filtered Tm
A1 65 Hi_NED Unknown Undet.
B1 65 Hi_NED Unknown Undet.
A1 65 IPC_VIC Unknown 32.108 0.601
B1 65 IPC_VIC Unknown 31.2587 0.601
A1 65 Nm Unknown Undet.
B1 65 Nm Unknown Undet.
A1 65 Spn_CY5 Unknown Undet.
B1 65 Spn_CY5 Unknown Undet.
Ready
Disconnected
NUM

Annex 3 Ethical approval

Norway

UNIVERSITETET I OSLO

DET MEDISINSKE FAKULTET

Dominique A. Caugant
Avd. for samfunnsmedisin
Universitetet i Oslo
Kirkeveien 166
0450 Oslo

Regional komité for medisinsk og helsefaglig forskningsetikk Sør-Øst C (REK Sør-Øst C)
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E-post: post@helseforskning.etikkom.no
Nettadresse: http://helseforskning.etikkom.no

Date: 14.09.2012
Your ref.:
Our ref.: IRB 0000 1870

To whom it may concern

With regards to the study *Characterization of pneumococcal isolates from patients in Ethiopia*

We hereby confirm that the Regional Committee for Medical and Health Research Ethics, section South-East C, Norway, has approved the project *Characterization of pneumococcal isolates from patients in Ethiopia* (Norwegian title: *Karakterisering av pneumokokkstammer isolert fra pasienter i Etiopia).*

The project was approved on the 16th of August 2012.

The ethics committee system consists of seven independent regional committees, with authority to either approve or disapprove medical research studies conducted within Norway, or by Norwegian institutions, in accordance with ACT 2008-06-20 no. 44: Act on medical and health research (the Health Research Act)

The ethics committee system consists of seven independent regional committees, with authority to either approve or disapprove medical research studies conducted within Norway, or by Norwegian institutions, in accordance with ACT 2008-06-20 no. 44: Act on medical and health research (the Health Research Act)

Please do not hesitate to contact the Regional Committee for Medical and Health Research Ethics, section South-East C (REK Sør-Øst C) if further information is required.

Yours sincerely,

Arvid Heiberg MD, PhD (sign.)
Professor of Medicine,
University of Oslo

Chair, Regional Committee
for Medical and Health Research Ethics,
section South-East C

Tor Even Svanes
Senior Advisor

Regional Committee for
Medical and Health
Research Ethics, section
South-East C

Annex 4 SERO Approval EHNRI,

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Ethiopian Health and Nutrition Research Instiutute
አዲስ አበባ
Addis Ababa,Ethiopia

E.H.N.R.I 6'13/954
Ref.No
ቀን 09 OCT 2012
Date

Mr. Surafel Fentaw
Principal Investigator
EHNRI

Subject: Approval of the Project Proposal

I would like to congratulate you and your group that your Research Proposal entitled "***Characterization of Pneumococcal Isolates from Patients in Ethiopia***" has been examined and approval for its Scientific and Ethical Review Committee.

We now advise you to start looking for funding if the funding is not already secured. Please note that the Institute will support the resource mobilization process as much as possible.

Working together for health!

Daddi Jima (MD, MPH)
Deputy Director General

CC:

- A/Director General's Office
- Infectious & non infectious disease Research Directorate
- Scientific and Ethical Review Office
  E.H.N.R.I

P.O.Box 1242/5654 Tel 0112138298/2753470 Fax 0112-75-86-34/757722 E-mail-daddij@ehnri.gov.et

ETHIOPIAN HEALTH AND NUTRITION RESEARCH INSTITUTE- (EHNRI)

SCIENTIFIC AND ETHICAL REVIEW COMMITTEE- (SERC)

PROJECT REVIEW DECISION FORM

PROJECT TITLE: Characterization of Pneumococcal isolates from patients in Ethiopia.

PRINCIPAL INVESTIGATOR: Mr. Surafel Fentaw

PROJECT NUMBER: SERO-110-1-2005

COMMENTS OF SERC

The above entitled project has been reviewed and was found to be scientifically valid and ethically clear. The outcome of the project is expected to provide evidence based information on the Characterization of pneumococcal isolates from patients in Ethiopia.

APPROVED [X] CONDITIONALLY APPROVED [ ] NOT APPROVED [ ]

SIGNATURE OF THE SERC MEMBERS

	Name	Signature
1.		
2.		
3.		
4.		
5.		
6.		

Date 05 / 10 / 2012

COMMENT AND FINAL DECISION OF THE INSTITUTE'S DIRECTOR

Accepted

APPROVED [X] CONDITIONALLY APPROVED [ ] NOT APPROVED [ ]

SIGNATURE _____ DATE 8/10/2012

Daddi Jima (MD, MPH)
Deputy Director General

Annex 5 Material Transfer Agreement (MTA)

MATERIAL TRANSFER AGREEMENT

From

The Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Abeba, Ethiopia

(herein referred to as ENHRI)

Represented by Dr. Almaz Abebe

To

WHO Collaborating Centre for Reference and Research on Meningococci, Division of Infectious Disease Control, Norwegian Institute of Public Health (NIPH), P.O.Box 4404 Nydalen, N-0403 Oslo, Norway **(herein referred as the RECIPIENT)**
Represented by Prof. Dominique A. Caugant

Materials:

Clinical specimens and pneumococcal isolates collected for the project entitled:
"*Characterization of pneumococcal isolates from patients in Ethiopia.*"

The Materials are provided to the RECIPIENT subject to the following terms and conditions:

1. The Materials are provided as a service to the research community and are to be used under the immediate and direct control of the RECIPIENT to determine the serotype, antibiotic resistance pattern, and characterize the molecular variations of pneumococcal strains causing disease in Ethiopia.
2. The Materials are not to be used: (a) in any product, (b) for the purpose of producing any product, or (c) for providing any service in which a product or service is sold or otherwise made commercially available. The Materials cannot be used for any other purpose without prior written consent of EHNRI.

3. The Materials are not to be made available to any other entity or researcher outside NIPH without prior written consent of EHNRI.

4. The source of the Materials should be acknowledged in any presentation or publication for which the Materials are used.

5. The Materials will be used in compliance with all applicable statutes and regulations, including regulations related to the disposal of hazardous materials.

folkehelseinstituttet

6. This Agreement shall terminate when the research noted in Section 1 is completed unless terminated or extended through written agreement of the parties. Either party may terminate this Agreement by giving sixty (60) days written notice to the other. The provisions of Section 3 shall survive termination of this Agreement.

We agree to the above terms and conditions:

Date: 21/9-2012

Signed on behalf of *NIPH* by:

Name: Dominique A. Caugant
Title/Position: Professor, Chief scientist

Signed on behalf of *EHNRI* by:

for 24 Sep /2012

Name: Almaz Abebe (PhD)
Title/Position: Directorate EHNRD